Biomolecular labelling for smFRET: Choosing dye pairs, placement and labelling approaches

Technical note

To perform a single-molecule Förster Resonance Energy Transfer (smFRET) experiment, biomolecules of interest must be labelled with a dye pair that can undergo FRET. The choice of dyes and their placement has a profound impact on the resultant data and therefore must be considered carefully when designing smFRET experiments[1,2].

Overview of this technical note:

- Discussion of various commercially available dye pairs and ideal characteristics for FRET
- Methods for site-specific labelling for proteins and nucleic acids
- Important considerations that influence how a dye pair may behave in different biomolecular environments or distances from one another

Common dyes used in smFRET experiments

An ideal fluorophore for smFRET can rapidly cycle between its excited singlet and ground states upon excitation, producing sufficient photons to enable accurate detection. Fluorophores should also exhibit photostability, whereby they are less likely to switch into a 'dark' triplet state or suffer photobleaching. Many next-generation fluorophores have been designed to produce the required brightness and photostability for single-molecule biophysical experiments[3]. It is important to choose dyes with spectral properties that are compatible with the lasers and optics of the instrument being used to collect the data. For the EI-FLEX and EI-FLEX Pro systems, two laser lines are used (most commonly 638nm with 450 or 520nm).

Common dyes used in smFRET experiments

Several dye pairs have been used in smFRET experiments to date, including ATTO dyes and Alexa Fluor dyes, and their activity is well-documented. These include:

- Cy3 and Cy5
- Alexa Fluor 550 and 647
- ATTO 550 and 647N

It is common for fluorophores in the green portion of the spectrum to be used as donors due to their favourable photophysical characteristics. They are usually bright and have good spectral overlap with fluorophores excited by the red end of the spectrum, which is essential for FRET to occur. Red fluorophores are therefore often good candidates for FRET acceptors, although they tend to suffer more from issues such as high hydrophobicity, so establishing compatibility with specific biomolecules of interest is key[2].

The image below illustrates the excitation and emission spectra for the dyes Cy3 and Cy5. Here, there is sufficient overlap between the emission spectrum of Cy3 and the excitation spectrum of Cy5, allowing FRET to occur. There is also some overlap between the two excitation spectra, meaning that Cy5 may be excited directly by the 520 nm laser; to read more about how to correct for this, see our technical note on accurate FRET correction.

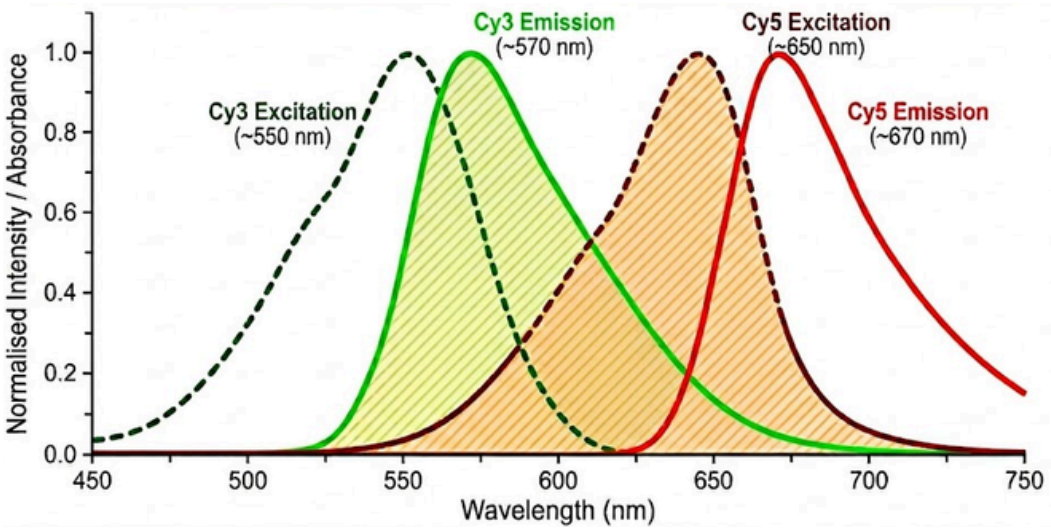

Figure 1 - Excitation and emission spectra for Cy3 and Cy5 fluorophores

Excitation spectra are indicated by dashed lines; emission spectra are indicated by solid lines. Hashed areas show overlap between Cy3 emission and Cy5 excitation wavelengths, where FRET can occur.
Note - spectra are illustrative, not exact.

Fluorescent proteins can also be used for smFRET. Although they are inherently fluorescent, there are several challenges associated with their use in smFRET experiments. They exhibit varying degrees of brightness and have broad excitation and emission spectra that lead to problematic levels of spectral overlap, although there are methods to mitigate this[4]. Fluorescent proteins are quite large relative to organic dyes, meaning that they take up much of the effective distance across which FRET can occur; max efficiencies tend to be around 40 %. They may also dimerise, reducing their suitability for interaction studies[5].

The impact of inter-dye distance and Förster radii

FRET efficiency is a measure of how effectively energy is transferred from a donor dye to a nearby acceptor dye. High FRET efficiency indicates that the labelled sites are closer to each other, while low FRET efficiency indicates they are further apart. The distance across which smFRET can occur is typically between 3 and 10 nm, although the specific pair used will determine the distances that can be accurately measured.
This is because the efficiency of the energy transfer between donor and acceptor is related to their specific properties and the distance between them, as described by the following equation[6]:

$$R = R_0 \times \sqrt[6]{\frac{1-E}{E}}$$

whereby:

R = distance between the fluorophores
R_0 = Förster radius (distance at which the FRET efficiency is 50 % for a given dye pair)
E = FRET efficiency

Figure 2 shows an example plot demonstrating how FRET efficiency is converted to distance for a Förster radius of 65 Å.

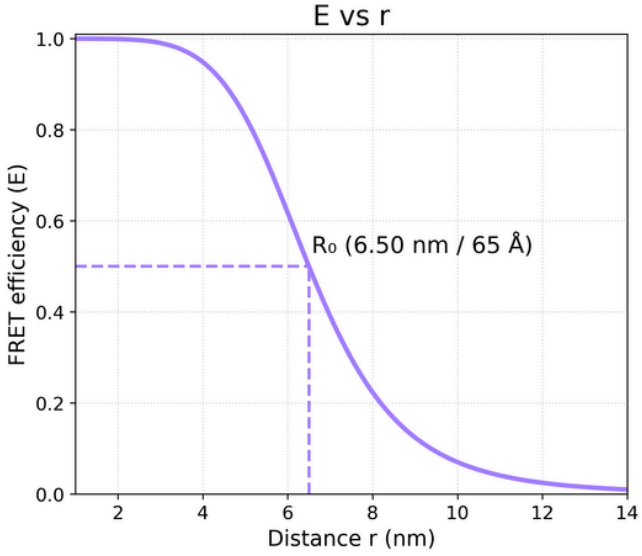

Figure 2 - FRET efficiency plotted against interdye distance

The impact of inter-dye distance and Förster radii

Förster radii are different for each dye pair, due to several photophysical factors[6]:

- **κ^2: the relative orientation of the donor and acceptor dipoles**
  - This value is often assumed to be ⅔ for freely rotating dyes, although if dye movement is restricted in some way, such as by steric hindrance or by sticking to a nearby residue, κ^2 values can change

- **The refractive index of the medium**
  - This can differ depending on the local environment in which dyes reside, affecting how efficiently donor dyes can excite acceptor dyes
  - Dyes freely diffusing in water versus those on the surface of a protein will result in different FRET efficiencies across the same distance

- **The quantum yield of the donor and the extinction coefficient of the acceptor**
  - These determine how many photons the donor emits upon excitation and how well the acceptor can absorb this light, respectively
  - Donor and acceptor pairs with higher quantum yields generally result in more efficient energy transfer and stronger emission signals

- **The spectral overlap of the dye pair**
  - This refers to how much of the donor's emission wavelength range overlaps with the acceptor's excitation wavelength range
  - A larger overlap permits measurements of FRET efficiencies over greater distances, but introduces challenges with increased likelihood of spectral bleed-through, and/or direct excitation of the acceptor

Therefore, although there are many published Förster radii for a range of dye pairs, it is important to consider how the factors above may impact Förster radii in the context of local environments for individual experiments.

It is typically recommended that if conformational changes are being measured by smFRET, optimal dye pair choices should result in the largest possible change in FRET efficiency between the hypothesised conformations. Therefore, predicted dye pair distances should ideally be centred around the Förster radius (or R_0) of the dye pair. This is because around R_0, small changes in the distance between donor and acceptor fluorophores result in large changes in FRET efficiency.

Dye pair placement considerations

The placement of dye pairs on biomolecules of interest must also be carefully chosen and optimised for individual systems. The distance between the dyes that result in meaningful measurements is limited by the fluorophores themselves, as discussed previously, but the biomolecule of interest that must be labelled also influences this decision.

It is crucial that labelling does not disrupt biomolecular function, such as causing steric hindrance at an active site, altering structural integrity, or causing aggregation. Fluorophore quenching from nearby residues should also be considered. It is recommended to utilise crystal structures and geometric simulations, such as Accessible Volume (AV) modelling (figure 3), to predict the spatial distribution of the fluorophores and their proximity to potentially quenching environments[2]. To read more on Accessible Volume (AV) modelling, see our technical note here.

Careful controls should also be performed to confirm native function before smFRET measurements can commence. Dye positions can always be re-evaluated if a particular dye position proves problematic. For example, while smFRET is a powerful technique for investigating IDPs, due to their highly dynamic nature, choosing labelling sites for these proteins can be more challenging.

Typically, crystal structures help identify surface-exposed residues that are likely to minimise disruption post-labelling. IDPs don't have a fixed structure and are highly sensitive to environmental factors, requiring careful planning and often the generation of multiple constructs to ensure that suitable labelling sites can be identified[7]. In a recent study, Riback et al. found that Alexa-488, a commonly used fluorescent label in smFRET studies, caused the well-characterised IDP PNt to undergo structural collapse, reducing FRET-resolved distances compared to those identified by small-angle X-ray scattering[8].

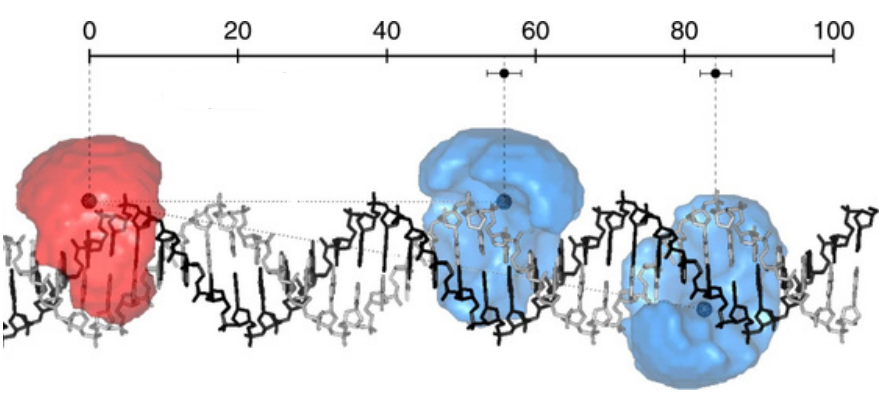

Figure 3 - Dye positions and AV modelling clouds on a double-stranded DNA molecule

Positions of a donor dye (red) and two acceptor dyes (blue) on a DNA duplex showing 'clouds' of all possible dye positions from AV modelling. Distances between dyes are given in Ångströms above.
Figure taken from Hellenkamp et al., 2018[9]

Common labelling methods

There are several methods for labelling proteins and nucleic acids with dyes for smFRET, as discussed below[3].

- **Cysteine-Maleimide reactions (via site-directed mutagenesis)**
    - This is the most common strategy, given that cysteines are a relatively rare amino acid. Site-directed mutagenesis is used to mutate all non-essential cysteine residues into another amino acid, such as alanine or serine, while inserting cysteines at the sites of interest
    - Next, maleimide-functionalised dyes are used to specifically label cysteine residues, targeting the thiol groups
        - For details on how to perform cysteine-maleimide labelling, we recommend this protocol[10]
    - If cysteine labelling is not suitable, amine labelling of lysines using N-Hydroxysuccinimide (NHS) esters is an alternative approach, although lysines are usually found in much higher abundance than cysteines
        - For details on how to perform amine labelling of lysines using NHS esters, we recommend this protocol[11]

- **Unnatural amino acids (UAAs)**
    - This is a slightly more challenging approach that involves in vitro synthetic protein production or encoding of the orthogonal tRNA and aminoacyl-tRNA synthetase pair via the Amber codon suppression strategy for *in vivo* expression
    - There are several UAAs that are used in this manner, containing reactive groups such as ketone, alkene or azide, the latter of which is common as it enables copper-catalysed azide–alkyne cycloaddition (CuAAC) click chemistry
    - Non-natural fluorescent amino acids can also be incorporated, such as 4-cyanotryptophan
    - An example of UAA labelling for smFRET can be found here[12].

Common labelling methods (continued)

- **Peptide tagging**
    - Proteins are engineered to express site-specific tags that can then react with dyes via click-chemistry or enzyme self-labelling (often used for in-cell labelling)
    - Several tags have been used for smFRET labelling as they produce minimal disruption to the local protein environment (these include HaloTags, CLIP-tags and SNAP-tags)
    - Another strategy is to use acyl carrier protein tags that incorporate CoA-conjugated fluorophores to serine residues (compatible with cell membrane labelling only)
    - Affinity-based non-covalent tagging can also be used, such as via His-tags
    - An example of how self-labelling enzymes were used to prepare proteins for smFRET can be found here[13].
- **Solid-phase synthesis**
    - This is the most common approach for nucleic acids around 100 bases or less, and involves site-specific incorporation of modified nucleotides via phosphoramidite chemistry
    - Dyes can also be incorporated post-synthesis, such as via the CuAAC click reaction
    - Modifications can be introduced into the backbone, sugars and bases themselves
    - Many companies synthesise fluorescently labelled nucleic acids, which can be used for smFRET experiments

Alternative methods for challenging biomolecules

While the methods above have been used successfully for a range of biomolecules, they may not be sufficient for labelling challenging samples. Recently, alternative methods have been published that aim to address these issues.

Labelling of long non-coding RNAs (lncRNAs)
Common methods for designing labelled nucleic acids only work for molecules up to 100 bases long. Therefore, this technique is unsuitable for lncRNAs, limiting smFRET research into key constructs such as riboswitches and ribozymes. Steffen et al. discuss a novel DNA-guided approach performed in three steps: reactive group activation, reactive group transfer, and dye coupling by CuAAC, which involves DNA helper and guide strands to bring the reactive groups into close proximity with chosen bases for labelling[14].

DNA scaffold method for labelling of an RNA pseudoknot
RNA pseudoknots are also challenging to label, given that many of the bases of interest may be buried within the secondary structure, while bulky dyes may disrupt pseudoknot formation and function. Graham et al. recently published a DNA-scaffold-based method that enables double labelling of an RNA pseudoknot, placing fluorescent dyes on the tip of stem 3 and the occluded 5' end. Briefly, the DNA scaffold is annealed, producing an RNA-DNA duplex, followed by Adenosine 5'-(γ-thio)-triphosphate and T4 polynucleotide kinase that permits incorporation of Cy3/5 maleimide following removal of the DNA strand by DNAases[15].

Summary

In summary, smFRET is a powerful technique for observing conformational dynamics and nanoscale distances for a range of biomolecules. When designing smFRET experiments, the local environment in which fluorescent dyes will be present, how they will be attached to the biomolecule of interest and the distance that needs to be measured should be taken into consideration.

For more information on the theory of smFRET and the capabilities of the EI-FLEX, see our Resource Library. Discover a range of applications for smFRET and the EI-FLEX platform on our website.

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